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(Problem 2 RWUD-1978)

FINAL REPORT

# A QUANTITATIVE STUDY OF THE YEASTS AND BACTERIA ASSOCIATED WITH LABORATORY-REARED SOUTHERN PINE BEETLE LARVAE

(Cooperative Agreement 19-288 with LSU-A)

J. Robert Bridges and James E. Marler

## SUMMARY

Frass and inner bark adjacent of larval galleries of laboratory-reared southern pine beetle, *Dendroctonus frontalis* Zimm., were sampled to determine the populations of yeasts and bacteria present at various stages of larval development. Yeasts were present in populations exceeding $10^6$ cells/mg and were present in higher numbers than bacteria by a factor of $10^2$ - $10^3$. Yeasts and bacteria were associated with all stages of larvae. We isolated filamentous fungi in large amounts only from galleries of later larval stages.

Of 25 species of yeasts identified from *D. frontalis* larval galleries, *Pichia pinus* (Holst) Phaff, *Candida tenuis* Diddens et Lodder, and *Hansenula capsulata* Wickerham were most frequently isolated. The high populations of yeasts associated with *D. frontalis* larvae supports the notion of other researchers that a symbiotic relationship exists between yeasts and bark beetles.

This study is closed.

UNITED STATES DEPARTMENT OF AGRICULTURE
FOREST SERVICE

SO-AL

REPLY TO: 4500 Forest Insect Research

April 5, 1982

SUBJECT: Final Report FS-SO-2203-1.42

TO: S. J. Barras, AD-W

Enclosed is a final report entitled A QUANTITATIVE STUDY OF THE YEASTS AND BACTERIA ASSOCIATED WITH LABORATORY-REARED SOUTHERN PINE BEETLE LARVAE.

Also enclosed is final report summary.

PETER L. LORIO, JR.
Project Leader
Forest Insect Research

Enclosures

PLLorio,Jr./do

cc: Bridges

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(Cooperative Agreement 19-288)

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## ABSTRACT

Frass and inner bark adjacent to larval galleries of laboratory-reared southern pine beetle, *Dendroctonus frontalis* Zimm., were sampled to determine the populations of yeasts and bacteria present at various stages of larval development. Yeasts were present in populations exceeding $10^6$ cells/mg and were present in higher numbers than bacteria by a factor of $10^2$ - $10^3$. Yeasts and bacteria were associated with all stages of larvae. We isolated filamentous fungi in large amounts only from galleries of later larval stages.

Of 25 species of yeasts identified from *D. frontalis* larval galleries, *Pichia pinus* (Holst) Phaff, *Candida tenuis* Diddens et Lodder, and *Hansenula capsulata* Wickerham were most frequently isolated. The high populations of yeasts associated with *D. frontalis* larvae supports the notion of other researchers that a symbiotic relationship exists between yeasts and bark beetles.

# INTRODUCTION

Although many species of microorganisms are associated with the southern pine beetle, *Dendroctonus frontalis* Zimm. (SPB), only three species of fungi are known to significantly affect beetle development. Two of these fungi are transported and propagated in the female's mycangium (Barras and Perry,1972). These mycangial fungi are obligate symbionts. In their absence SPB progeny development is delayed, and brood production is decreased by more than 60% (Barras, 1973).

The third fungus is the bluestain fungus, *Ceratocystis minor* Hedgc. It is also consistently associated with the SPB (Nelson and Beal, 1929, Rumbold,1931) and affects the beetle's development. This fungus contributes to tree death which apparently aids beetle development by speeding desication of the tree (Bramble and Holst,1940, Nelson and Beal,1929, Nelson,1934). It has also been suggested that *C. minor* favors larvae development by creating a more favorable nutritional source (Clark and Richmond, 1977).

The importance of the other microorganisms found with the SPB is not known. Previous studies have identified several species of bacteria from the gastrointestinal tracts of adult SPB and from their galleries (Oberle, 1966; Moore, 1972). Yeasts are also associated with bark beetles (Callaham and Shifrine, 1960), but their role in larval development is not known.

Studies to date have identified some of the microorganisms associated with the SPB but have provided no quantitative information on the populations of these microorganisms present at various times. Our purpose in this experiment was to obtain a quantitative estimate of the populations of bacteria and yeasts in SPB larval galleries and to identify those microorganisms present in the greatest numbers. We used bolts inoculated in the laboratory so we could better control some of the conditions which might affect the gallery microflora. We wanted to study those organisms carried by SPB and not be confused by microorganisms possibly carried by associated insects. Also, in laboratory-inoculated bolts the galleries were less crowded which facilitated sampling of individual larval galleries without interference from nearby galleries.

## MATERIALS AND METHODS

Emergent SPB were collected from naturally infested loblolly pine trees (*Pinus taeda* L.) using a collection box similar to the one described by Browne (1972). Beetles were separated by sex and introduced into pre-formed holes in freshly cut loblolly pine bolts in the laboratory. The ends of the bolts were sprayed with White's solution (Barras, 1972), and each beetle establishment point was sterilized with flaming alcohol. A plug of bark was removed with a 10-mm diameter bark punch. One female and one male beetle was placed in each hole and covered with a thin piece of bark. Sterile paraffin was used to seal the plug of bark of each inoculation hole. Bolts were kept under ambient conditions in the laboratory.

Bolts were sampled at weekly intervals when they contained predominantly the following SPB stages: 1) Eggs; 2) Young larvae in mines before they had begun to form feeding chambers; 3) Mid-stage larvae feeding in larval chambers; 4) Outer bark larvae.

Samples were taken at approximately the midpoint of the egg gallery. At each stage of development 3 egg galleries (replications) were sampled. Outer bark was aseptically removed to expose the gallery, and phloem tissue was sampled from around eggs or larvae. Three larval galleries were sampled for each replication. Samples from the 3 larval galleries were pooled and weighed together. For each larval gallery 4 samples were taken--one sample of frass from the egg niche and 3 samples of phloem (Fig. 1). Phloem samples included the gallery wall. In galleries containing eggs and very young larvae, sampling positions B and C (Fig. 1) were in advance of gallery construction. In these cases samples were taken at distances from the egg niche to approximate where the larval gallery would be. Samples were taken using a 2-mm cannula as a bark punch. Samples were weighed and then thoroughly homogenized in 1 ml of glucose, peptone, yeast extract (GPYE) medium (see below) using a glass tissue grinder. Serial dilutions to $10^{-4}$ were made in GPYE, and 0.1-ml a aliquot was spread on each of the isolation media.

The following culture media were used: 1) GPYE (Glucose, peptone, yeast extract)--Prepared by mixing together 2 separately autoclaved solutions. Solution I: $K_2HPO_4$ , 6.3 g; $NaH_2PO_4 \cdot H_2O$, 1.9 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.008 g; Ferric citrate, 0.008 g; Bacto yeast extract (Difco), 5.0 g; $H_2O$, 950 ml. Solution II: 50 ml of a 20% glucose solution. 2) BHI-Brain heart infusion (Difco). 3) ME--2.5% Difco malt extract. 4) WA--Difco wort agar. Liquid media were solidified with 2% agar.

Plates were incubated for 5 days at 28°C. Either all colonies or representative colonies were picked from the highest dilution plates. Therefore, microorganisms isolated and identified from each sample were primarily those organisms present in the greatest numbers.

Yeasts were identified using the methods of Lodder (1970) and Barnett and Pankhurst (1974). Bacteria were identified using standard procedures (MacFaddin, 1976; Buchanan and Gibbons, 1974).

Data on mean numbers of yeasts or bacteria per mg of wet weight of tissue were analyzed by an analysis of variance. Data were transformed using a square root transformation before analysis. A split plot design was used to evaluate differences in the number of microorganisms due to stage of development of the larva or position in the larval gallery. Stage of development was the whole plot factor, and sample position was the sub-plot factor.

## RESULTS AND DISCUSSION

A complete list of the yeasts and bacteria isolated in this study is given in Table 1. We isolated approximately equal numbers of fungi on ME and on WA. More bacteria were isolated on BHI than on the other media. By experimental design the isolated organisms were primarily those present in the highest numbers in each sample and are not meant to be a complete list of all organisms in each sample.

*Pichia pinus* was the most frequently isolated yeast. Of the 48 samples (3 replications x 4 stages x 4 gallery positions), *P. pinus* was isolated from 20 of the samples (Table 1). *Candida tenuis, Hansenula capsulata, H. polymorpha,* and *Pichia* sp. were also frequently isolated. *P. pinus* and *C. tenuis* were isolated from all stages of development.

*Pichia pinus* and *H. capsulata* have been isolated from SPB (Brand *et al.*, 1977; Barras and Perry, 1972) and are two of the most frequently isolated yeasts from bark beetles (Shifrine and Phaff, 1956; Callaham and Shifrine, 1960). *Candida tenuis* has also been isolated from SPB (Oberle, 1966) and other bark beetles (Callaham and Shifrine, 1960). Most of the other yeasts have been isolated from bark beetles or forest environments (Callaham and Shifrine, 1960; Lodder, 1970).

We found fewer species of bacteria than yeasts (Table 1), although many other bacteria can be isolated from the SPB using enrichment cultures.[1/] *Enterobacter agglomerans* was isolated from 14.6% of the samples. *Enterobacter agglomerans* has been isolated from *D. terebrans* Olivier, and some of the isolates fixed nitrogen (Bridges, 1981). One of the isolates from this study was tested, but it did not fix nitrogen.

One of the purposes of this study was to determine whether the microorganism populations in larval galleries changed as the larvae developed. We found a large amount of variability in the abundance of microorganisms among the replicates. More yeast than bacterial cells (by a factor of $10^2$ - $10^3$) were isolated regardless of stage of development or sampling position (Table 2). Differences in yeast or bacterium populations among various stages of larval development were not significant. There were significantly more yeast cells per mg of wet weight in egg niche frass samples than in phloem samples. Phloem samples probably contained more moisture than frass samples which could account for the lower yeast counts in phloem. There were significantly more yeast cells isolated from the egg niche phloem than from the other phloem samples.

[1/] Marler, J. E. and S. J. Barras. 1978. Identification of bacterial flora in galleries of the southern pine beetle, *Dendroctonus frontalis* Zimmerman. Final Report, U. S. Forest Service, FS-SO-2203-1.29, 19pp.

Although this study was not designed to quantify filamentous fungi we isolated several species especially during the later stages of development. For example, on ME we found filamentous fungi only at the later 2 stages of development (Table 2). In addition to the two species of mycangial fungi (Barras and Perry, 1972) and *C. minor*, *Trichoderma* sp. and *Penicillium* sp. were commonly isolated. These results suggest that a succession of microorganisms occurs in SPB galleries with yeasts and bacteria being quantitatively more important in early galleries. A similar succession has been noted in pine trees attacked by *Dendroctonus ponderosae* Hopkins (Robinson, 1962). Microbial successions are well documented for decaying wood systems (Blanchette, 1979).

However, our results are not conclusive. Our failure to isolate filamentous fungi on dilution plates probably indicates that the filamentous fungi were not sporulating in early galleries. Although each yeast or bacterium cell is a propagule, the number of propagules of filamentous fungi would vary with the number of spores present or the ability of a particular fungus to be homogenized and remain viable.

Our results show that a wide variety of organisms, especially yeasts, are associated with SPB larval galleries. The species of organisms varied with replication as well as with sample position and stage of development of the larvae. From a quantitative standpoint there does not appear to be a specific yeast or bacterium associated with SPB. Other workers have found that although an individual bark beetle may have only one yeast associated with it, the individuals of a species vary in their yeast associates (see Callaham and Shifrine, 1960).

## CONCLUSIONS

Our results with laboratory-reared SPB confirm that yeasts are consistently associated with SPB and are quantitatively more important than bacteria. High populations of yeasts are present with SPB throughout its development.

A symbiotic [1/] relationship exists between bark beetles and yeasts, but the exact nature of the relationship is not known. The constant association of yeasts with bark beetles has led some to suggest that yeasts are obligate sybionts (Callaham and Shifrine, 1960; Whitney 1971; Farmer, 1965).

By quantifying the populations of yeasts associated with laboratory-reared SPB, we provide additional evidence for an obligate symbiosis. The high population of yeasts associated with SPB suggest that yeasts affect beetle development, but the role that yeasts might have in beetle development is not known.

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1/ We use symbiosis in its broadest sense as the living together of dissimilar organisms, regardless of the possible results of the association (Leach, 1940).

Yeasts could affect the insect directly by supplying nutrients (such as vitamins) or by degrading potentially toxic host tree compounds. Yeasts may also produce bark beetle behavioral chemicals (Brand *et al.*, 1976; 1977; Callaham and Shifrine, 1960). Yeasts may affect SPB development indirectly by their association with the mycangial fungi. Mutualism between yeasts and bluestain fungi associated with *D. ponderosae* has been suggested by Whitney (1971). A mutalistic association among yeasts and wood-decaying fungi has previously been reported in decay studies of conifers (Blanchette and Shaw, 1978).

To determine the role of yeasts, studies are needed of SPB development in the presence or absence of yeasts and other symbiotic fungi. Based on the results of this study, *P. pinus*, *C. tenuis*, and *H. capsulata* would be candidate organisms for such a study.

## REFERENCES

Barnett, J. A. and R. J. Pankhurst. 1974. A New Key to the Yeasts. North-Holland Publishing Co., Amsterdam.

Barras, S. J. 1972. Improved White's solution for surface sterilization of pupae of *Dendroctonus frontalis*. J. Econ. Entomol. 65: 1504.

Barras, S. J. 1973. Reduction of progeny and development in the southern pine beetle following removal of symbiotic fungi. Can. Entomol. 105: 1295-1299.

Barras, S. J. and T. Perry. 1972. Fungal symbionts in the prothoracic mycangium of *Dendroctonus frontalis* (Coleopt.:Scolytidae). Z. angew. Entomol. 71: 95-104.

Blanchette, R. A. 1979. A study of progressive stages of discoloration and decay in *Malus* using scanning electron microscipy. Can. J. For. Res. 9: 464-469.

Blanchette, R. A. and C. G. Shaw. 1978. Associations among bacteria, yeasts, and basidiomycetes during wood decay. Phytopath. 68: 631-637.

Bramble, W. C. and E. C. Holst. 1940. Fungi associated with *Dendroctonus frontalis* in killing shortleaf pines and their effect on conduction. Phytopath. 30: 881-899.

Brand, J. M., J. W. Bracke, L. N. Britton, A. J. Markovetz, and S. J. Barras. 1976. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. J. Chem. Ecol. 2: 195-199.

Brand, J. M., J. Schultz, S. J. Barras, L. J. Edson, T. L. Payne, and R. L. Hedden. 1977. Bark beetle pheromones: enhancement of *Dendroctonus frontalis* (Coleoptera:Scolytidae) aggregation pheromone by yeast metabolites in laboratory bioassays. J. Chem. Ecol. 3: 657-666.

Bridges, J. R. 1981. Nitrogen-fixing bacteria associated with bark beetles. Microb. Ecol. 7: 131-137.

Browne, L. E. 1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. J. Econ. Entomol. 65: 1499-1501.

Buchanan, R. E. and N. E. Gibbons (ed.). 1974. Bergey's Manual of Determinative Bacteriology, 8th edition. The Williams and Wilkins Co., Baltimore.

Callaham, R. Z. and M. Shifrine. 1960. The yeasts associated with bark beetles. For. Sci. 6: 146-154.

Clark, E. W. and J. A. Richmond. 1977. Variations of free and triglyceride fatty acids in phloem of *Pinus taeda* infected by *Ceratocystis minor*. Turrialba 27: 377-383.

Farmer, L. J. 1965. The phloem-yeast complex during infestations of the mountain pine beetle in lodgepole pine. Ph.D. Thesis. Univ. of Utah.

Leach, J. G. 1940. Insect Transmission of Plant Diseases, 1st edition. McGraw-Hill Book Co., New York. 615 p.

Lodder, J. (ed.) 1970. The Yeasts: A Taxonomic Study, 2nd edition. Lubrecht and Cramer, Monticello, NY.

MacFaddin, J. F. 1976. Manual of Biochemical Tests for Identification of Medical Bacteria. Williams and Wilkins Co., Baltimore.

Moore, G. E. 1972. Microflora from the alimentary tract of healthy southern pine beetles, *Dendroctonus frontalis* (Scolytidae), and their possible relationship to pathogenicity. J. Invert. Path. 19: 72-75.

Nelson, R. M. 1934. Effect of bluestain fungi on southern pines attacked by bark beetles. Phytopath. Z. 7: 327-352.

Nelson, R. M. and J. A. Beal. 1929. Experiments with bluestain fungi in southern pines. Phytopath. 19: 1101-1106.

Oberle, A. 1966. The microflora isolated from galleries of southern pine bark beetles, *Dendroctonus frontalis* Zimm. M. S. Thesis. Northwestern State College, Natchitoches, LA.

Robinson, R. C. 1962. Blue stain fungi in lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) infested by the mountain pine beetle (*Dendroctonus monticolae* Hopk.). Can. J. Bot. 40: 609-614.

Rumbold, C. T. 1931. Two blue-staining fungi associated with bark beetle infestation of pines. J. Agric. Res. 43: 847-873.

Shifrine, M. and H. J. Phaff. 1956. The association of yeasts with certain bark beetles. Mycologia 48: 41-55.

Whitney, H. S. 1971. Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. Can. Entomol. 103: 1495-1503.

Table 1. Yeasts and bacteria associated with SPB larval galleries at various stages of development.

| Microorganism | Frequency of isolation (%) | Occurrence at each stage of development: Egg | Young larva | Mid-stage larva | Larva in outer bark |
|---|---|---|---|---|---|
| YEASTS | | | | | |
| *Pichia pinus* (Holst) Phaff | 41.7 | + | + | + | + |
| *Candida tenuis* Diddens et Lodder | 37.5 | + | + | + | + |
| *Hansenula capsulata* Wickerham | 31.2 | + | + | - | + |
| *H. polymorpha* De Morais et Maia | 22.9 | + | + | + | + |
| *P.* spp. | 22.9 | + | + | - | + |
| *Torulopsis ernobii* Lodder et Kreger-Van Rij | 12.5 | + | - | + | + |
| *H. philodendra* Vander Waltet Scott | 12.5 | - | + | - | + |
| *H.* spp. | 8.3 | + | + | - | + |
| *P.. toletana* Socias, Ramirez et peaez | 8.3 | - | - | + | - |
| *H. holstii* Wickerham | 6.2 | + | + | + | - |
| *C. silvanorum* Vanderwalt, Scott et vander Klift | 6.2 | - | + | + | + |
| *C.* spp. | 6.2 | - | + | - | + |
| *Saccharomyces bisporus* Naganishi | 6.2 | + | - | - | + |

Table 1. (Cont.)

| Microorganism | Frequency of isolation (%) | Occurrence at each stage of development: Egg | Young larva | Mid-stage larva | Larva in outer bark |
|---|---|---|---|---|---|
| YEASTS (Cont.) | | | | | |
| *S. rouxii* Boutroux | 6.2 | - | - | - | + |
| *P. bovis* Van Uden et do Carmo-Sousa | 6.2 | - | - | + | - |
| *Torulopsis* spp. | 4.2 | - | + | + | - |
| *Trichosporon cutaneum* DeBeurm, Gougerot et Vaucher | 4.2 | - | - | - | + |
| *Saccharomyces* spp. | 4.2 | - | - | - | + |
| *C. rugosa* (Anderson) Diddens et Lodder | 2.1 | - | + | - | - |
| *P. nonfermentans* Nakase | 2.1 | - | + | - | - |
| *H. henricii* Wickerham | 2.1 | - | + | - | - |
| *C. valida* Leberle | 2.1 | - | + | - | - |
| *C. quercuum* Nakase | 2.1 | - | - | + | - |
| *C. terebra* Suqiyama ct Goto | 2.1 | - | - | - | + |
| *C. humicola* (Daszewska) Diddens et Lodder | 2.1 | - | - | - | + |
| *C. lusitaniae* Van Uden et do Carmo-Sousa | 2.1 | - | - | + | - |

Table 1. (Cont.)

| Microorganism | Frequency of isolation (%) | Occurrence at each stage of development: Egg | Young larva | Mid-stage larva | Larva in outer bark |
|---|---|---|---|---|---|
| YEASTS (Cont.) | | | | | |
| *Cryptococcus laurentii* Kufferath | 2.1 | - | - | + | - |
| *T. milischiana* Zikes | 2.1 | - | - | + | - |
| BACTERIA | | | | | |
| *Micrococcus luteus* (Schroeter) Cohn | 29.2 | + | + | + | + |
| *Bacillus* spp. | 29.2 | + | + | + | + |
| *Enterobacter agglomerans* (Beijerinck) Ewing and Fife | 14.6 | + | + | + | - |
| Nocardia "rhodochrous group" 1/ | 12.5 | - | + | - | + |
| *Acinetobacter calcoaceticus* (Beijerinck) Baumann, Doudoroff et Stanier | 8.3 | + | - | + | + |
| *Streptococcus faecalis* Andrewes et Horder | 2.1 | - | - | + | - |
| *Bacillus brevis* Migula | 2.1 | - | - | - | + |

Table 1. (Cont.)

| Microorganism | Frequency of isolation (%) | Occurrence at each stage of development: Egg | Young larva | Mid-stage larva | Larva in outer bark |
|---|---|---|---|---|---|
| BACTERIA (Cont.) | | | | | |
| *Micrococcus* sp. | 2.1 | - | - | - | + |

1/ Formerly *Mycobacterium rhodocrous*

Table 2. Mean number of yeasts, bacteria, and filamentous fungi isolated from SPB larval galleries per mg of wet weight of tissue.

| Gallery sampling position | Developmental Stage[1] | | | |
|---|---|---|---|---|
| | Egg | Young larvae | Mid-stage larvae | Larvae in outer bark |
| | Yeast cells/mg (x $10^5$)[2] | | | |
| Egg niche frass | 0.4(0.4) | 7.1(2.5) | 4.5(2.5) | 5.2(3.3) |
| Egg niche phloem | 0.1(0.1) | 2.2(0.3) | 2.5(1.4) | 1.9(1.3) |
| Larval mine phloem | 0 | 1.4(0.9) | 0.04(0.04) | 0.4(0.3) |
| Larval chamber phloem | 0 | 1.3(1.2) | 0.2(0.2) | 0.4(0.2) |
| | Bacterium cells/mg (x $10^3$)[2] | | | |
| Egg niche frass | 0.6(0.6) | 47.2(2.5) | 144.8(128.0) | 51.6(45.8) |
| Egg niche phloem | 1.4(1.4) | 5.8(4.8) | 74.4(71.1) | 0.05(0.05) |
| Larval mine phloem | 0 | 1.5(1.5) | 0 | 1.0(1.0) |
| Larval chamber phloem | 0 | 0.2(0.2) | 0 | 0 |
| | Fungus propagules/mg (x $10^3$)[2] | | | |
| Egg niche frass | 0 | 0 | 0.2(0.2) | 176.5(129.2) |
| Egg niche phloem | 0 | 0 | 0.1(0.1) | 4.6(4.3) |
| Larval mine phloem | 0 | 0 | 0.8(0.4) | 0.02(0.02) |
| Larval chamber phloem | 0 | 0 | 0 | 1.4(1.3) |

[1]Numbers in parentheses are standard errors of the mean.

[2]Yeasts and filamentous fungi were isolated on ME; bacteria were isolated on BHI.

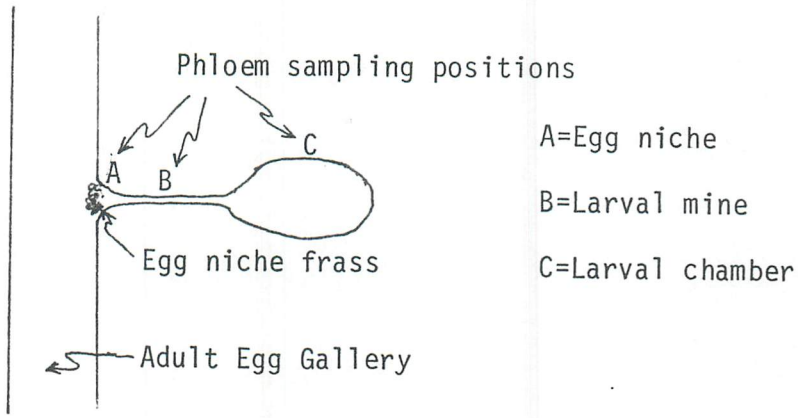


Figure 1. Diagram of SPB larval gallery showing sampling positions.